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An *in vitro* test of the efficacy of an anti-biofilm wound dressing

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ABSTRACT

Broad-spectrum antimicrobial agents, such as silver, are increasingly being formulated into medicated wound dressings in order to control colonization of wounds by opportunistic pathogens. Medicated wound dressings have been shown *in-vitro* to be effective against planktonic cultures, but *in-vivo* bacteria are likely to be present in biofilms, which makes their control and eradication more challenging. Recently, a functional wound dressing (AQUACEL® Ag+ Extra™ (AAg + E)) has been developed that in addition to silver contains two agents (ethylenediaminetetraacetic acid (EDTA) and benzethonium chloride (BC)) designed to disrupt biofilms. Here, the efficacy of AAg + E is demonstrated using a biofilm model developed in an isothermal microcalorimeter. The biofilm was seen to remain viable in the presence of unmedicated dressing, silver-containing dressing or silver nitrate solution. In the presence of AAg + E, however, the biofilm was eradicated. Control experiments showed that neither EDTA nor BC alone had a bactericidal effect, which means it is the synergistic action of EDTA and BC disrupting the biofilm with silver being bactericidal that leads to the product's efficacy.

1. Introduction

The process of healing of chronic cutaneous wounds is complex and may be affected by the presence of microorganisms (Bowler, 2002). In particular, bacterial contamination may slow wound healing, resulting in damage to surrounding tissue and ultimately infection of the host (Landis, 2008). Progression to infection may also be aided by other factors including poor blood supply to the wound and the intrinsic virulence properties of the invading organisms (Siddiqui and Bernstein, 2010; Bowler et al., 2001).

The fact that the treatment of bacterial infection at the wound site has the potential significantly to reduce the time for wound healing has led to the development of a number of medicated wound dressings containing antimicrobial agents. Silver is a particularly widely used agent, as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms, Miraftab et al., 2014) and anti-fungal activity (Bowler et al., 2005), although there is debate as to the specific efficacy of silver (Aziz et al., 2012; White and Cutting, 2006) and to its potential toxicity (Hermans, 2006).

An added complexity in the treatment of chronic wound infections is that the organisms are frequently present as biofilms, with this form of bacterial growth being increasingly implicated in cases of poor wound healing (Hurlow and Bowler, 2012). Evidence for biofilm involvement in chronic wounds comes from macroscopic (Hurlow and Bowler, 2012; Metcalf and Bowler, 2013) and microscopic observation of biofilms (James et al., 2008; Metcalf and Bowler, 2013). A study involving *in vivo* wound models of a *Staphylococcus aureus* infection also showed microscopic evidence of biofilm formation along with a demonstration of physiological differences between planktonic cells and biofilm bacteria recovered from wounds (Davis et al., 2008).

While the drivers for biofilm formation are not completely clear, experimentally it is usually observed that microbial biofilms show reduced antimicrobial sensitivity than comparable plankonic organisms (Davis et al., 2008; Percival et al., 2011). Antibiotic resistance has been ascribed to several mechanisms, including the production of inactivating enzymes, the presence of persister cells and the protective effects of extracellular polymeric substances (EPS) (Smith, 2005).

Successful treatment of chronic wound infections therefore requires development of next generation of medicated dressings that are efficacious against biofilms. AQUACEL® Ag+ Extra™ (AAg+E) is a recently developed dressing that contains two agents that are known to disrupt biofilms, ethylenediaminetetraacetic

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acid (EDTA) and benzethonium chloride (BC), in addition to ionic silver as an antimicrobial agent. A recent cohort analysis of wound healing data involving 121 clinical cases showed AAg+E to result in progress toward healing in over 90% of wounds which were previously stalled, infected or at a risk of infection and with a high suspicion of biofilm contamination (Metcalf et al., 2014).

Determination of wound dressing efficacy using analytical or traditional microbiological methods is tricky, because of the challenges inherent in determining viable bacterial counts in a heterogeneous system. Isothermal microcalorimetry (IMC) is one technique that offers potential in this area, since it can detect the power resulting from bacterial growth without requiring optical clarity of the sample. We showed previously how IMC can be used to quantitate wound dressing efficacy (Gaisford et al., 2009; Said et al., 2014) against planktonic cultures of two common wound pathogens. To use IMC to investigate the efficacy of AAg+E against biofilms, however, requires development of a biofilm model. Hence, the specific aim of this work was to develop a biofilm model suitable for use with IMC and to use the model to explore the efficacy of AAg+E.

2. Material and methods

Ethylenediaminetetraacetic acid (EDTA), benzethonium chloride (BC) and silver nitrate ($AgNO_3$) were purchased from Sigma (UK) and used as received. Wound dressings, AQUACEL® (AH), AQUACEL® Ag (AAgH) or AQUACEL® Ag Extra (AAg+E) were supplied by ConvaTec Ltd. The wound dressings, all comprised of sodium carboxymethylcellulose fibers, differ in that AH has no antimicrobial agent while AAgH contains ionic silver and AAg+E contains ionic silver, EDTA and BC.

The challenge organism, *S. aureus* NCIMB 9518, was grown overnight in nutrient broth (NB; Oxoid Ltd.) for 16 h at 37 °C. Cells were then harvested, washed in phosphate buffered saline (PBS), resuspended in 15% v/v glycerol at an organism density of 10^8 cfu/ml and frozen in aliquots (1 mL) over liquid nitrogen (Beezer et al., 1976; Cosgrove, 1979). Aliquots were stored under liquid nitrogen until required. Previous experience (data not shown) has indicated that organisms can be stored for over 6 years in this frozen state and remain viable post thawing with less than 1% decrease in viability.

Ampoules were prepared by adding molten agar (300 μL, Agar No. 3, Oxoid), which was allowed to set. Tryptone soya broth (TSB, 1 mL) was added to prepared ampoules and inoculated with *S. aureus* to a final population density of 1×10^6 cfu/mL. The ampoules were then sealed and incubated at 37 °C with agitation for 24 h. Thereafter, the ampoules were opened and the TSB was carefully poured out. The ampoules were gently rinsed with sterile distilled water a minimum of five times to ensure effective removal of planktonic cells. Fresh, sterile TSB (1 mL) and where required wound dressing (3.3 mg), EDTA (0.14% w/v), BC (0.4% w/v) or $AgNO_3$ (1×10^{-4} M) was then added to the ampoules, which were hermetically sealed and placed into calorimeters.

Cell counts were determined after each calorimetric experiment. After removal of TSB and extensive rinsing, as above, fresh PBS was added to ampoules, which were then sealed. Ampoules were then briefly vortexed before being sonicated for a period of 5 min. Colony counts were determined by serially diluting the PBS from the ampoules and spread plating onto iso-sensitest agar. Colonies were counted following 16 h incubation at 37 °C.

Calorimetric data were recorded with a 2277 Thermal Activity Monitor (TAM; TA Instruments Ltd., UK) operated at 37 °C. Data capture was initiated exactly 30 min post inoculation with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier setting 300 μW). The instrument was calibrated periodically by the electrical substitution method. Data were analyzed using Origin 8.1 (Microcal Software Inc.).

3. Results and discussion

Although not discussed here, it was not possible to culture a biofilm directly onto the glass walls of the ampoule. Rather, the agar layer was necessary to encourage biofilm attachment and growth. A similar effect has been noted in ecological research where a twofold increase in the accumulation of diatoms (phytoplanktonic algae) was seen on a surface when unenriched agar was added (Stevenson, 1983). The rationale in that study was the use of agar to 'simulate mucilage of immigrating organisms', in other words to create a 'slimy', polymeric conditioning film. In relation to *in vivo* correlation it has been suggested that the use of agar simulates a microbial colonization of a moist surface, similar to wounds (Daeschlein et al., 2010).

The physiology of cells in biofilms is distinct from those in planktonic culture because of their sessile nature and differences in exposure to nutrients and gases in their microenvironment (Stewart and Franklin, 2008). Diffusion is the main mechanism of delivery of solutes to biofilm clusters (Stewart, 2003) and so concentration gradients exist that depend on the thickness of the biofilm. It follows that delivery of antimicrobial agents will similarly be rate limited by this diffusion mechanism, and their efficacy may be reduced relative to planktonic cultures (up to a 1000 fold decrease in susceptibility has been reported, Stewart and Costerton, 2001).

Similarly, the power produced by an established biofilm will differ from that of a planktonic culture. As has been discussed in the literature (Braissant et al., 2010; Said et al., 2014) the power output from planktonic cultures will show a series of exothermic peaks and troughs with time as the organisms utilize the available nutrients to increase in number. Integration of the power data with respect to time gives a plot of cumulative heat versus time, which is comparable to the standard growth curves determined with optical density (OD) readings.

Typical power-time data for *S. aureus* biofilms are shown in Fig. 1. Biofilms were found not to give as repeatable data as planktonic cultures. Similar variability was seen in a study by Astasov-Frauenhoffer et al. (2012). However, the degree of variability was small enough to permit study of the efficacy of the dressing. It is apparent that there is an exothermic peak

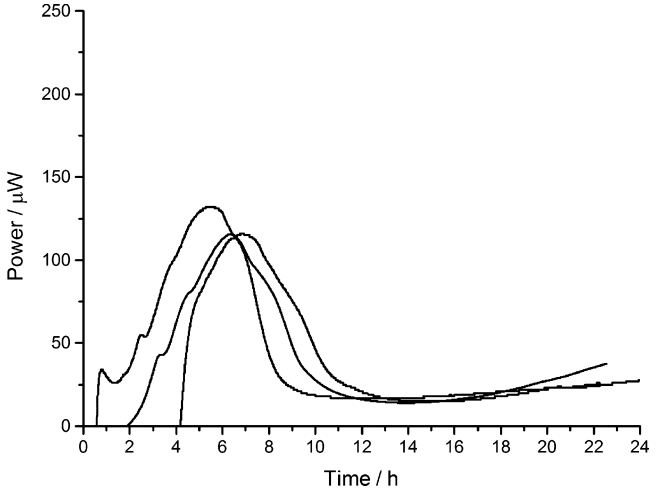

Fig. 1. Power-time data for biofilms grown on agar, showing the level of reproducibility for three samples.

initially, followed by a smaller, but persistent, exothermic power. It is assumed that the initial peak represents either growth of planktonic cells released from the biofilm or growth and metabolism within the biofilms themselves. The power then resolves to a non-zero baseline, which is assumed to be associated with biofilm metabolism.

The presence of the initial exotherm is surprising. Following 24 h incubation, the putative biofilm samples were extensively rinsed in order to remove planktonic cells. Each rinsing can be considered as a 1:1 dilution as the volume of diluent used was equivalent to that of the original growth medium in the culture. As such, very little or no power, or at least a significant delay in onset of power signals, would be expected from planktonic cells as these would have been diluted down to undetectable levels (given that the lower limit of detection for IMC is approximately 1×10^5–1×10^6 cfu/mL, Said et al., 2014).

Cell counts for biofilm samples suggest a potential explanation. Immediately following preparation (i.e., before being loaded into the calorimeter) the average cell count was 4×10^7 cfu/mL. After 24 h in the calorimeter, the average cell count had increased to 1×10^8 cfu/mL. This suggests that cell division continues within the biofilm, presumably until the available nutrients and/or gases in the fresh medium are exhausted, after which the system reverts to a stasis metabolism. An alternative explanation is that since rinsing will disturb the isotonic balance of the system, the biofilm responds by dispersing bacteria from the film as a survival response. These bacteria then grow as a planktonic population.

The corresponding cumulative heat plot for the biofilm is shown in Fig. 2 (all further data are shown as cumulative heat plots for familiarity and ease of comparison with OD data).

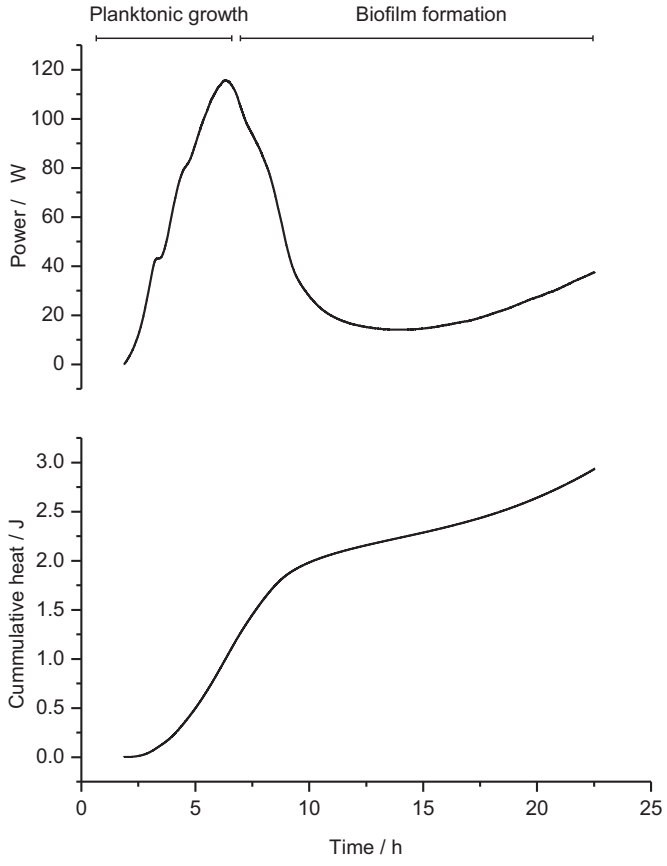

Fig. 2. Power-time data for the control biofilm (bottom) and corresponding growth curve (top).

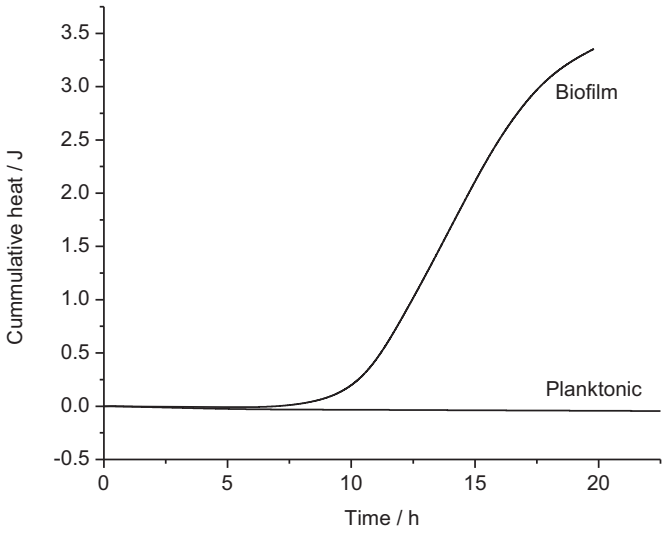

Fig. 3. Growth curves for planktonic and biofilm cultures in the presence of 1×10^{-4} M $AgNO_3$.

Previously Said et al. (2014) showed that $AgNO_3$ at a concentration of 1×10^{-4} M was bactericidal against planktonic *S. aureus*. Fig. 3 shows the growth curves for planktonic and biofilm cultures in the presence of this silver concentration. As expected, no growth is seen in planktonic culture, but the biofilm culture shows growth following an initial delay. The average cell count following the calorimetric experiment was 1.5×10^7 cfu/mL, not much lower than that recorded for the untreated biofilm, which confirms that biofilm formation confers on the participant organisms a significant degree of protection against antimicrobial agents.

The implication of this finding is that a wound dressing containing silver alone might be less effective against a biofilm culture, and this effect is indeed observed experimentally, Fig. 4. Dressing alone (AH) did not inhibit growth, while silver-containing dressing (AAgH) only delayed growth (the same effect noted above in the silver nitrate experiments).

Successful eradication of an established biofilm requires delivery of agents to disrupt biofilm structure in addition to an

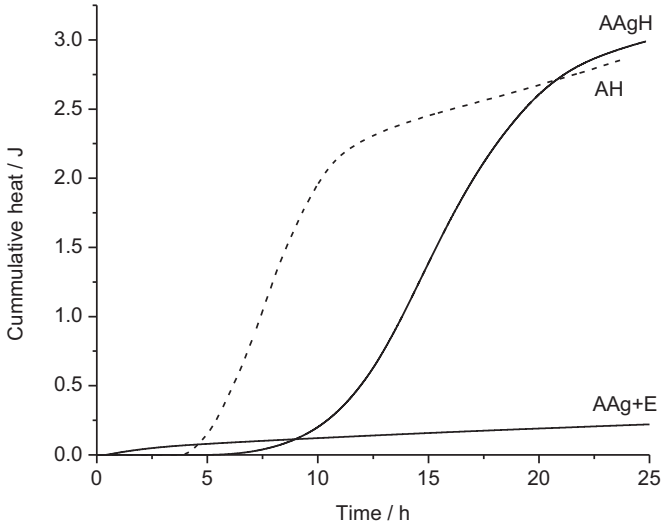

Fig. 4. Growth curves for biofilms in the presence of AH, AAgH and AAg+E.

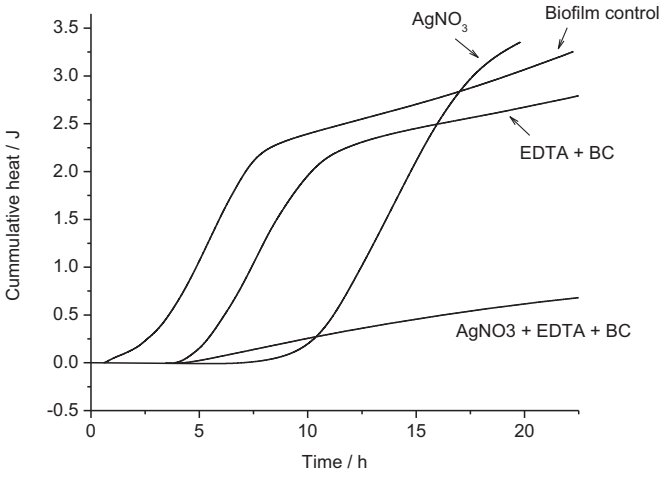

Fig. 5. Growth curves for biofilm alone and in the presence of $AgNO_3$, EDTA + BC and $AgNO_3$ + EDTA + BC.

antimicrobial compound. This is the formulation strategy behind AAg+E and the data in Fig. 4 show a significant reduction in growth with this dressing. The average cell count following exposure to AAg+E was 2×10^4 cfu/mL, so the dressing can be deemed to be bactericidal in nature because it induced a 3 log reduction in viable cells relative to the control.

The question then remains as to the relative contributions of the components in AAg+E to the overall efficacy of the dressing. Biofilms were exposed to solutions containing only $AgNO_3$, only EDTA and BC or all three components (Fig. 5). In all cases a small amount of AH (i.e., plain dressing) was included, the intention being to imitate the action of a dressing containing only these components. It is apparent that EDTA and BC cause an initial delay but then growth is seen which is identical to the control. We noted previously that the presence of AH can alter the kinetics of growth (Said et al., 2014), possibly because the hydrofiber network can sequester planktonic bacteria, changing the rate of supply of nutrients and altering local pH. It is also possible that the hydrofiber itself can cause physical disruption of a biofilm. An effect on growth rate in the presence of AH is seen in Fig. 4. Silver nitrate is seen to inhibit growth for at least 10 h, after which growth similar to the control is seen. In neither case therefore do these components act to inhibit the biofilm. When they are added in combination, however, a reduction in growth is seen, which confirms the hypothesis that it is the combination of biofilm disrupters and an antimicrobial agent working in synergy that is necessary for efficacy.

The effect of EDTA has been attributed to its properties as a metal chelator, since the chelation of divalent cations that are important in ensuring the structural integrity of cells causes disruption and prolonged exposure may result in a biocidal effect (Banin et al., 2006). Certain divalent cations have also been indicated in the structural integrity of the biofilm EPS matrix (Banin et al., 2006; Percival et al., 2005) and sequestration of these cations may result in dispersal of the biofilm.

EDTA may also play a role in the delivery of silver to the bacteria. Since silver is contained in the dressing in its ionic form, delivery to its site of action will be affected both by the concentration of free silver in solution and the relative association constants between silver and components in solution and the bacterial cell wall. It is likely that binding to the EDTA affects the relative positions of these association constants, and since the silver appears to have faster action in the presence of EDTA the data imply the association constant of silver with EDTA is fast, ensuring silver ions are rapidly removed from the dressing, but not as strong as the association constants of silver with the cells.

BC, on the other hand, is a surfactant. In high concentrations BC has antiseptic and anti-infective properties that have been shown to have anti-biofilm activity against oral fungal biofilms (Ichikawa et al., 2008), but the concentrations achieved from AAg+E are below the MIC. Thus, BC acts to reduce surface tension, affecting biofilm architecture and cell–cell interactions (Davey et al., 2003), promoting the activity of silver when delivered with EDTA.

4. Summary

A biofilm model was developed that enabled the use of IMC to test the efficacy of an anti-biofilm wound dressing. It was shown that a broad spectrum antimicrobial agent alone was not effective against the biofilm but that when biofilm disrupting agents were included in the dressing bactericidal action was seen.

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